THE CLEARANCE OF HETEROLOGOUS PROTEIN FROM THE CIRCULATION OF NORMAL AND IMMUNIZED MAN [1, 2]

By ANTHONY P. FLETCHER, NORMA ALKJAERSIG, AND SOL SHERRY

(From the Medical Section, The Research Institute of the Jewish Hospital of St. Louis, and the Department of Medicine, Washington University School of Medicine, St. Louis, Mo.)

(Submitted for publication November 13, 1957; accepted June 5, 1958)

The clearance rates of intravenously administered antigenic protein from the blood stream of normal and immunized animals have been studied by a number of observers and the results have been reviewed by Coons (1). Proteins labeled in various ways were used during these investigations, but the most relevant results appear to have been obtained when proteins were trace-labeled with radioisotopes (2), a technique resulting, under the best conditions, in little or no detectable change in the biological fate of the protein. In most instances the experimental conditions were such that the rate of protein removal was gradual and the period of observation extended over several days. However in one recent study (3) particular attention was paid to the changes occurring in the first hour or so after the injection of the antigenic material into the immune animal, a time during which antigen-antibody union in the circulation might be expected to exert a marked influence upon the clearance rate. Virtually all previous investigators have used the rabbit as a test animal and studies in man have not been reported.

Streptokinase, a product of hemolytic streptococcal metabolism, when injected into man by the intravenous route and at appropriate dosage, will cause the activation of serum plasminogen and the occurrence of a fibrinolytic state (4). During the course of work aimed at inducing a state of controlled fibrinolysis in man for therapeutic reasons (5), the opportunity arose to determine the plasma clearance rates of streptokinase from the circulation of normal and immune man. Most streptococcal proteins are antigenic to man and streptokinase is no exception; consequently as a result of naturally occurring hemolytic streptococcal infections the population exhibits a variable level of specific antibody toward this material. Indeed, the injection of streptokinase will induce in almost all, if not all, subjects and after a latent period a rapid sustained rise of specific antibody. Despite the antigenic nature of streptokinase the intravenous injection of highly purified preparations (6) is tolerated without clinical or other evidence of deleterious effect (7).

It is perhaps a misnomer to talk of unimmunized man in connection with streptokinase as this is an ubiquitous antigen; probably few humans constitute virgin soil with regard to it. Nevertheless, this term is used to indicate those persons who show either very low or no circulating levels of specific antibody. Moreover, one outstanding advantage accrues from the use of this system since it enables studies to be carried out on a population which has been subjected to an antigenic stimulus of a "normal" intensity, rather than a population that has received an intense "unphysiological" stimulus. Thus the mechanisms disclosed may be pertinent to the problems encountered under human disease conditions. Furthermore, in all previous studies, doses of several mg. antigen N per Kg. body weight have been used, whereas in this study it has been possible to use only a few micrograms antigen N per Kg. body weight. The use of these much smaller doses might be expected to yield results more immediately relevant to the course of events following the common types of immunological insult in man.

The present communication describes the findings following the intravenous administration of isotopically labeled streptokinase into normal (*vide supra*) and immunized man. The results indicate that the elimination of antigenic protein from the blood stream of man parallels those mechanisms

[1] This work was supported by grants from the National Heart Institute, United States Public Health Service, Bethesda, Md., and Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

[2] Presented before the Thirtieth Annual Meeting of the Central Society for Clinical Research, Chicago, November 1 and 2, 1957.

described as occurring in the rabbit, though certain significant species differences do exist.

METHODS

Two methods were used to prepare streptokinase to be used for labeling. Material prepared by the first method (6) assayed at 600 streptokinase units per microgram N was biophysically homogeneous, but showed the presence of several antigenic components on immunochemical analysis; material prepared by the second method [3] was of similar potency and similarly homogeneous, by biophysical examination, but was markedly superior to the other on immunochemical analysis (8). Both materials reacted similarly to iodination procedures and both were treated identically following *in vivo* injection. The majority of the work was performed using material prepared by the second method.

The labeling technique was based on that of Eisen and Keston (9), the reaction pH being 8.8. An ion exchange column (Amberlite® IRA-400) was used to remove uncombined iodine. The eluate was taken to 50 per cent ammonium sulphate saturation and the precipitate dissolved in normal saline containing 0.05 M phosphate buffer at pH 7.5 with 1 per cent human serum albumin. Sterilization was by Seitz filtration (Swiney model), the pad being saturated by 1 per cent human albumin prior to use and being washed with 5 ml. of 1 per cent albumin after the streptokinase had been passed.

The streptokinase recovery after the resin stage was 85 to 100 per cent of the original activity, but usually a 10 to 30 per cent loss occurred during filtration as a result of the small amount of protein being handled (1 mg.). The amount of free I^{131}, as determined after trichloracetic acid precipitation, was under 3 per cent. The integrity of the iodination bond was tested by running paper electrophoretic strips and preparing radioautographs. The labeled material ran with the same mobility as unlabeled streptokinase and the radioautographs could be superimposed on the protein strips. Control determinations with I^{131} mixed with streptokinase showed the radioactivity to travel independently of the protein band and labeled material mixed with free I^{131} showed two bands on the radioautographs. The degree of I^{131} tagging varied with different preparations, but assuming a molecular weight of 60,000 for streptokinase (6) the degree of iodination produced in the batches used for human tracer studies varied from 0.2 to 0.7 atoms of I^{131} for each molecule of streptokinase. Thus the streptokinase used was trace-labeled rather than converted into heavily iodinated protein, a step which would have resulted in spurious clearance rates (10). It is apparent, assuming that the molecular weight for streptokinase is correct, that one atom of iodine may be substituted into each streptokinase molecule without loss of biochemical activity. However data on the effect of a higher degree of iodination are lacking. More heavily iodinated batches usually lost biochemical activity, but the effect was inconstant and was probably attributable to the differing chemical treatment of the proteins, rather than to the alteration of their structure.

Radio-activity was determined in a well-type scintillation counter with an efficiency of approximately 50 per cent. Blood samples withdrawn for assay were oxalated, centrifuged and the plasma counted. The protein was then precipitated by adding 2 ml. of 15 per cent trichloracetic acid to 3 ml. of plasma and centrifuging off the precipitate. Control runs with unbound I^{131} added to the plasma showed that over 95 per cent could be recovered in the supernatant and that iodinated protein was totally precipitable. All plasma counts, except where otherwise noted, refer to protein bound activity rather than to total plasma activity and all counts were corrected back to the original volumes. Clearance studies were made with 4 to 15 microcuries of activity. So as to facilitate comparisons between clearance studies performed with different batches of labeled material of varying radioactivity, clearance plots have been expressed as streptokinase units per ml. plasma, rather than as cpm per ml. plasma. Counting of centrifuged red cell concentrates revealed that no greater activity was found than could be accounted for by the entrapped plasma content and that this activity could be removed by washing. No increase of free I^{131} was found after labeled streptokinase had been incubated with blood or plasma for 12 hours at 37°C.

Streptokinase was assayed as previously described (6) and the biochemical activity expressed in Christensen units (11). Streptokinase antibody assays were performed upon the isolated gamma globulin fractions of serum prepared by the technique of Lever and co-workers (12) and assayed by a modification of the Christensen technique (11). Clearance studies were performed on patients who required intravenous streptokinase for therapeutic reasons. Assay samples were withdrawn from the arm other than the one that had been used for the streptokinase injection or infusion.

Relation of the specific antibody content of plasma to its total streptokinase inhibitory power. The dose of streptokinase required to induce whole blood fibrinolysis in man can be predicted by means of a simple *in vitro* test (the predicted dose test) that provides a measure of the total inhibitory effect of plasma against streptokinase (13). This figure, expressed in units streptokinase per ml. plasma, when multiplied by the patient's calculated plasma volume indicates the total streptokinase dosage to produce fibrinolysis. The inhibitory action of plasma upon streptokinase is a consequence of two mechanisms. First, since antiplasmin will inhibit plasmin, a threshold streptokinase concentration must be exceeded before detectable plasminogen activation occurs and second, as a consequence of immunization the plasma may contain streptokinase antibody.

It has been necessary to establish the relative importance of these two mechanisms in our patients at different levels of plasma inhibitory action; otherwise the use of a figure for total plasma antibody would have had only

[3] Kindly supplied by Dr. J. Ruegsegger, Lederle Laboratories, Pearl River, N. Y.

TABLE I

The antibody content of plasma related to the total inhibitory effect of plasma upon streptokinase

Total inhibitory effect of plasma on streptokinase units / ml.	Average and range (in %) of inhibition due to antibody content of plasma	No. of observations
0–10	51 (37–68)	7
10–20	74 (58–97)	8
20–200*	86 (70–121)	7

* The techniques are less accurate at the highest levels of inhibition. As the degree of plasma streptokinase inhibition increases the greater is the proportion of inhibition contributed by specific antibody.

doubtful validity. Table I shows the comparison between the total inhibitory effect of the plasma (predicted dose test) and the fraction resulting from the presence of antibody. It is apparent that not only is the concept of the total plasma antibody valid, but that antibody accounts for the major part of the inhibition shown by our patients and that this mechanism is of increasing importance as the total inhibitory power of the plasma increases. Furthermore the occurrence of fibrinolysis must indicate that an excess of antigen has been infused.

Throughout this communication the value for total plasma antibody has been derived by multiplying the antibody content per ml. of plasma by the plasma volume calculated from the patient's body weight, and the demonstration of plasma or whole blood fibrinolysis accepted as evidence of antigen excess in the plasma.

Nature of the streptokinase-antibody complex. Specific antigen-antibody precipitates were never demonstrable despite the use of many immune sera and the trial of many antigen/antibody ratios, nor was isotopically labeled streptokinase precipitated by immune sera. Probably the failure to demonstrate precipitating antibodies rests on a quantitative rather than a qualitative basis, since calculation indicates that the quantity of streptokinase antibody found, even in hyper-immune sera, may be below the amount required to yield a detectable specific precipitate. However recent work using gel diffusion techniques suggests that precipitating antibodies against streptokinase may be demonstrable in both immune animal and immune human sera (8). Despite this latter finding it is clear that under the experimental conditions used, the antigen-antibody complexes formed were, at least *in vitro,* always of the soluble variety. Presumably a similar situation obtained *in vivo.*

RESULTS

Plasma clearance studies in man

Figure 1 (A) illustrates the rate of labeled streptokinase clearance from the circulation of a 56 year old man who received 100,000 units over a five minute period by the intravenous route. *In vitro* testing had shown him to have total plasma antibody equivalent to 29,000 streptokinase units. The rate of initial clearance is seen to be extremely rapid, but following the removal of a portion of the labeled material the clearance rate becomes much slower. Figure 1 (B) shows a precisely similar experiment in a 42 year old man, except that *in vitro* testing had shown him to have a total plasma antibody equivalent to 61,000 units. The curves in Fig. 1 (A) and (B) are seen to be of a similar form but to differ in respect to their proportions. Clearly a greater amount of the injected material has been removed rapidly in the latter experiment where the amount of circulating antibody was the greater. Analysis of these curves by a regression method (3) shows that each is composed of two exponential clearance components, the 50 per cent clearance rates for the first component being respectively 17 and

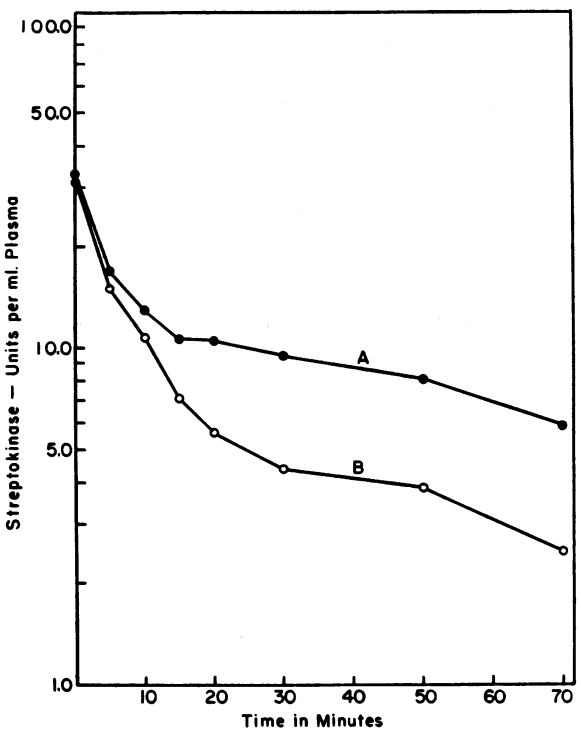

FIG. 1. THE PLASMA CLEARANCE RATE OF LABELED STREPTOKINASE

The plasma streptokinase concentrations were obtained after the injection, at zero time, of labeled streptokinase (100,000 units) in Patients A and B. The total plasma antibody, determined before the injection of streptokinase, was 29,000 units in Patient A and 61,000 units in Patient B.

TABLE II

Rate of streptokinase clearance from the circulation at various antigen/antibody ratios *

ANTIGEN DEFICIENCY			ANTIGEN EXCESS		
Total circulating antibody	Antigen/Antibody (in %) injected prior to clearance study	50% plasma clearance in minutes	Total circulating antibody	Antigen/Antibody (in %) injected prior to clearance study	50% plasma clearance in minutes
29,000	complex curve**	11	29,000	complex curve**	62
61,000	complex curve**	17	61,000	complex curve**	83
1,000,000	5	14	200,000	150	60
150,000	30	20	38,000	150	87
70,000	70	15	120,000	130	70
50,000	60	36	50,000	170	81
90,000	20	9	50,000	900***	92
65,000	90	15	80,000	1,000***	101
			65,000	1,400***	110

* Antibody assays were made prior to streptokinase therapy. Clearance studies during conditions of antigen excess (right half of the table) were made using less than 10,000 units of labeled streptokinase.

** Derived from data of Figure 1.

*** Clearance rates determined during the second day of therapy.

11 minutes and the 50 per cent clearance rates for the second component being 62 and 83 minutes. Since all streptokinase clearance curves performed under conditions of antigen excess show these two components, the first component has been designated as "fast" and the second as "slow." These designations employed throughout the text have been expressed as 50 per cent plasma clearance rates in minutes.

Though regression analysis of complex curves provides much information, it was felt to be preferable to study simple exponential clearance rates since the requisite information could be obtained with fewer analyses and thus fewer samples would have to be withdrawn from the patient. Since the fast rate of plasma clearance appeared to occur when circulating antibody was either in excess or equivalence with the antigen, six clearance studies were performed on individual patients utilizing less antigen than was required to combine with the total plasma antibody. In all instances the material was cleared at a simple exponential rate with an average 50 per cent clearance time of 17.5 minutes. Details of six experiments and the two earlier experiments are shown in Table II and the actual experimental findings for two patients are illustrated in Figure 2. It is to be noted that whatever the initial level of plasma antibody and whatever the proportion of injected antigen in relation to this last figure, the clearance rate remained approximately the same and, provided that antibody remained in excess, antigen clearance was very fast.

Unlabeled streptokinase in a dose at least 30 per cent in excess of that required to combine with all the circulating antibody was injected into seven patients. Brisk fibrinolysis was induced in all and following the production of this state, labeled material was injected and its plasma clearance determined. Two of the patients used were those whose fast rates of clearance are illustrated in Figure 2 and the results of the second clearance determination in these patients are also illustrated in Figure 2. Of the remaining five patients, the second clearance was determined in three during the second day of therapeutic treatment after they had received a large excess of antigen. The details of these seven clearance determinations are shown in Table II; the average 50 per cent rate of clearance under these circumstances was 83 minutes. The two rates of clearance, the fast and the slow, differ significantly (t equals 13.7 and $p < 0.001$) and the only explanation for this difference appears to lie in the presence of circulating antibody in the plasma during the determination of the first clearance rate and its absence during the determination of the second rate.

Evidence from long duration streptokinase infusions

Calculation from the data of Table II shows that to maintain a steady streptokinase concen-

tration of 10 streptokinase units per ml. plasma during the "slow" clearance phase approximately 30,000 streptokinase units per hour should be infused. However this calculation being based on short term plasma clearance studies probably offers inadequate correction for antigen removal by antibody union in the extravascular spaces. Accordingly, four patients whose total plasma antibody varied between 35,000 to 70,000 units received a dose of labeled streptokinase 50 per cent in excess of their total antibody. Commencing 30 minutes later, when the "fast" rate of clearance would be largely complete, further labeled streptokinase was administered at the rate of 50,000 units per hour for 8 hours. Serial determinations of plasma streptokinase concentrations were made during and after the infusions. Three of the four patients showed gradually rising plasma streptokinase concentrations peaking at 8 to 10 streptokinase units per ml. at the end of the infusion, but the fourth patient showed a sharper rise, his highest concentration being 18 units per ml. Thus in all the patients, during the eight hour period, the elimination rate of streptokinase from the circulation was less than, but not much less than, 50,000 streptokinase units per hour. This compared well with the calculated figure of 30,000 units per hour. However, one further patient who had a much higher total plasma antibody, 150,000 units, showed different behaviour. The experiment was conducted similarly except that he received 100,000 units per hour; his plasma streptokinase levels rose more slowly than those of the other patients and peaked at 6 units per ml. This difference was attributed to extravascular antigen fixation. Concomitant biochemical studies showed that in each patient the plasma streptokinase concentration was correlated with plasma fibrinolytic activity measured on preformed I^{131} labeled plasma clots (5).

Experience with the infusion of unlabeled streptokinase (65 infusions in 40 patients) has provided confirmatory evidence of our findings with labeled streptokinase. Empirically we have found that patients with an initial plasma antibody below 50,000 units maintain a relatively steady

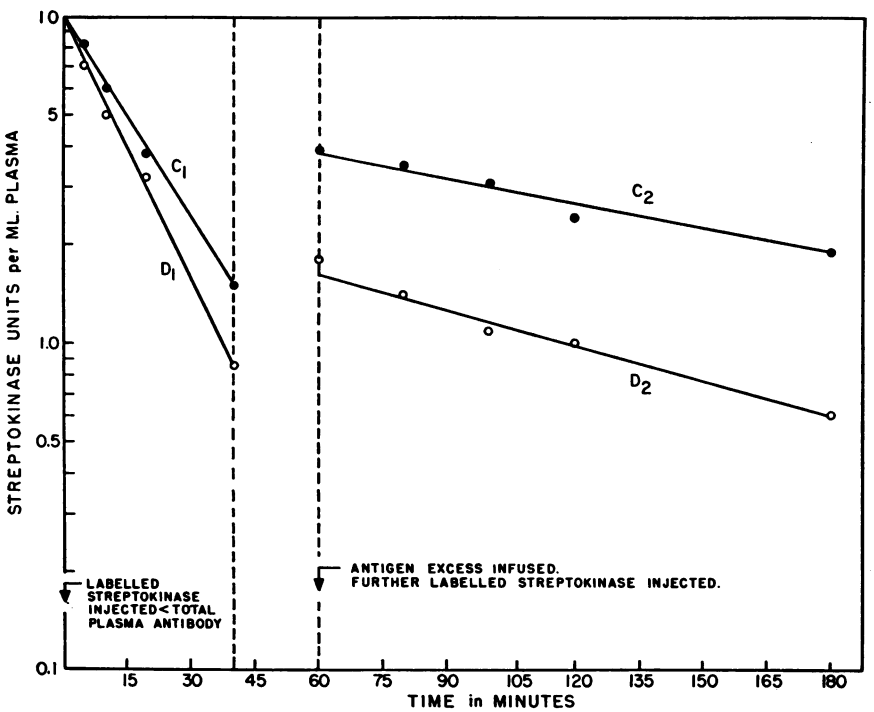

FIG. 2. THE "FAST" AND "SLOW" STREPTOKINASE PLASMA CLEARANCE RATES IN TWO PATIENTS

The patients cleared the injected streptokinase at the "fast" rate from 0 to 40 minutes and at the "slow" rate from 60 minutes onward.

fibrinolytic state with 35,000 units per hour, patients with an initial antibody of 50,000 to 100,000 units require 50,000 units per hour, whereas those with higher initial plasma antibody totals usually need 100,000 units per hour. Obviously, under these experimental circumstances, the actual initial plasma antibody content has no direct relevance to the mechanism of antigen clearance and is being used solely as a measure of the patient's immunological status towards streptokinase. Fifteen of these patients received infusions lasting 30 to 40 hours and in all there was clear biochemical evidence that the clearance rate of streptokinase remained at least relatively constant throughout the infusion period. The fibrinolytic activities assayed were similar to those recorded with labeled streptokinase, slowing of the infusion rates caused a reduction in the biochemical activities and within two to three hours of stopping the infusion all fibrinolytic activity disappeared. Since the apparent space distribution of human albumin at equilibrium has been found to be 2.5 times the plasma volume (14), these long infusion periods exclude intravascular-extravascular equilibration as being the cause of the present finding.

Breakdown of the antigen and organ localization

Catabolism of the labeled material was rapid, 22 to 55 per cent of the injected I^{131} being excreted in the urine in 24 hours, when doses of 4 to 15 microcuries were injected. All the urinary I^{131} was in dialysable form and at the end of this period a substantial thyroid uptake of I^{131} was demonstrable; this I^{131} was assumed to have been taken up by the thyroid after degradation of the protein molecule. Unbound I^{131} could be detected in the plasma shortly after the injection of the labeled material and the proportion of unbound to protein bound I^{131} showed an increase with time; usually the free I^{131} was 3 to 6 per cent of the total during the first 45 minutes. Later, however, the ratio of free to protein bound I^{131} increased; by 6 hours free I^{131} was 20 to 30 per cent of the total and by 24 hours, 40 to 50 per cent of the total. The significance of these later readings is somewhat uncertain as the protein bound component may not represent labeled streptokinase.

External monitoring with a directional scintillation detector was performed in two patients. The somewhat higher counts recorded over the liver and spleen were suggestive rather than conclusive evidence of antigen localization within these organs.

Change in the quantitative ability of the human to clear injected antigen at the fast rate following antigenic stimulation

Since the "fast" clearance rate involves the combination of antigen and antibody, the increase of circulating antibody following specific antigenic stimulation should increase the ability of man to clear injected antigen at the "fast" rate. This effect was demonstrated in two patients who received streptokinase therapy and as a consequence became immunized.

The first patient was shown on the basis of *in vitro* testing to require 30,000 units of streptokinase to induce fibrinolysis; he received 50,000 units intravenously and fibrinolysis was produced. Immediately following the onset of fibrinolysis, labeled streptokinase was injected and the 50 per cent clearance rate was shown to be 92 minutes; that is, the material was cleared at the "slow" rate. The patient then received 1.2 million units of streptokinase over 3 days, and 10 days later he had become immunized to the extent that *in vitro* testing indicated that he would require a dose of 300,000 units to induce fibrinolysis. He received 200,000 units intravenously and his blood failed to exhibit the phenomenon of fibrinolysis; an immediate clearance with labeled material showed that streptokinase was being cleared at the fast rate (50 per cent clearance in 14 minutes). A second patient, who received 2.4 million streptokinase units over three days and became immunized, was tested in a like manner and essentially similar results were recorded. Thus the high plasma antibody level following immunization had resulted in a markedly increased ability to clear streptokinase at the "fast" rate. A patient who had received streptokinase, but who had not become immunized, showed no increased ability to clear injected antigen by the "fast" rate.

Clinical effects of the antigen injections

The usual expectation following the intravenous injection of antigen into immunized man or animals is that a degree of clinical upset will occur

amounting in the severer cases to anaphylactic shock or even death. Not infrequently a subsequent episode of "serum sickness" may result.

It has been recently reported that immune man tolerates without substantial clinical disturbance the infusion of streptokinase in doses of 2 to 5 micrograms antigen N per Kg. body weight over a four hour period (7). However in the present work doses of up to 10 micrograms antigen N per Kg. body weight have been injected over a 10 minute period without detectable clinical disturbance in immune man. Furthermore, following the use of initial large doses to remove circulating antibody, maintenance of the desired intense fibrinolytic state (5) has required the infusion of 0.75 to 2.5 micrograms antigen N per Kg. body weight per hour for periods of 30 or more hours. Deleterious clinical effects attributable to the antigen injection have been absent and serum sickness has not subsequently developed though on occasion, mild hemorrhagic phenomena related to the intensity and duration of the fibrinolytic state have been noted (5). Thus man appears tolerant of this antigen (at least within the stated dose limits) by intravenous injection during states of antibody excess, antigen-antibody equivalence and vast antigen excess.

DISCUSSION

It is apparent that in man, as in the animal, there exist two rates of plasma clearance for antigenic proteins from the blood stream. If specific antibody is involved the antigen is cleared at a relatively great rate, but if specific antibody is absent a much slower rate of clearance occurs.

The following reasons are held to justify the reference in the text to the streptokinase inhibitory material as antibody. *1*) The inhibitory material is present in the gamma globulin fraction. *2*) Streptokinase administration causes, after a latent period, a great rise in the serum gamma globulin capacity for streptokinase inhibition. *3*) The length of the latent period is variable, but consistent with that found with other antibody systems and on the whole is shorter when the initial level of inhibitory material is high (booster effect). *4*) Following the latent period, the rise of inhibition is sustained for many months. *5*) The concentrations of serum inhibitory material are distributed in the population as a log-normal variable—the common type of distribution found for antibody levels.

The clearance results obtained were not attributable to the denaturation of the labeled streptokinase by the iodination process, as each batch of labeled material was shown to be cleared at either the fast or slow rate, under appropriate circumstances. No batch of labeled material used in man suffered loss of specific biochemical activity and the biological testing showed that each individual batch of material yielded essentially similar results. However extrapolation of the plasma clearance lines to determine the theoretical plasma concentration at zero time and using a plasma volume calculated from the patient's weight showed that frequently the estimated zero time plasma concentration of labeled material was only 80 per cent of the calculated theoretical concentration. This suggested that a minor portion of the injected antigen could have been cleared in a manner different from that of the major portion. This hypothesis receives support from the work of McFarlane (15), who compared the clearances of C^{14} (biologically labeled) and I^{131} trace-labeled proteins in rabbits and found that sometimes a portion of the I^{131} trace-labeled protein was removed in a manner different to that of the C^{14}-labeled proteins. He showed that this discrepancy could be corrected by "filtering" the I^{131}-labeled protein in the circulation of an animal. However it is unlikely that serious error was introduced by this failure to account for all the injected material as the discrepancy was comparatively small and the anomaly was only evident in the moments required for equilibrium prior to the withdrawal of the first sample.

It is improbable that stripping of the protein label, apart from true protein catabolism, played any significant role in the results, though Freidberg, Walter and Haurowitz (16) have shown that this may be a serious source of error in determining concentrations of tissue antigens after a period of days. First, clearance rates were measured for only two to three hours; secondly, only plasma clearance rates rather than tissue concentrations were measured; and thirdly, control experiments showed that the label remained protein bound when the labeled streptokinase was incubated with plasma.

No method is at present available for the direct determination of streptokinase in plasma and as a consequence quantitation of plasma streptokinase rested solely upon assay for the radioactive label. However, indirect biochemical evidence, evinced through measurement of the components of the plasminogen-plasmin system, has been consistent with the hypothesis that the label and biochemical activity remained associated under *in vivo* conditions. Though it has been shown in man that I^{131} trace-labeled human albumin, prepared by a variety of techniques, may be cleared at different rates from the body (14), these differences were only detectable over a period of days and the present experiments were conducted over a period of minutes. Moreover there is *in vitro* evidence that labeling with I^{131} may leave the immunological properties of an antigen unchanged (9) and *in vivo* evidence that the ratio of label to immunological activity of the protein remains constant for a period of days (17, 18). Lastly there is recent evidence in the rabbit that the anomalies in I^{131} label clearances previously reported were a function of the technique used rather than of the method itself (15, 19).

The "fast" rate of plasma clearance appears to result as a consequence of antigen-antibody union and the removal of this complex. It is of peculiar interest that, under these experimental circumstances, the antigen-antibody complex remains in the soluble form without precipitation, in fact the clearance mechanism is of such an effective nature that it is difficult to conceive of a situation in which a precipitating antigen-antibody complex could be formed. This situation appears to be wholly different from that obtaining in the rabbit where the occurrence of the "fast" clearance is apparently dependent upon the formation of a precipitating complex (3). Since, under these circumstances in man, the concentration of antigen and antibody does not need to approach the precipitating concentrations in order for the fast clearance rate to occur, it would seem likely that this fast clearance of antigen may be found to occur universally in immunized man and may thus be a biological phenomenon of the widest significance.

The "slow" rate of plasma clearance had an average half time of 83 minutes and showed a wide variation. None of the material appears as antigen in the urine. Breakdown rates, as far as they could be judged by determining the ratios of free and protein bound I^{131} in the plasma and the excretion of this material in the urine, appeared to be equally high for material cleared by either the "fast" or the "slow" rate. Though these rates may have been influenced by the presence of extravascular antibody, the high antigen/antibody ratios used in some experiments exclude this factor as a major source of error.

Most of the patients in whom clearance studies were performed suffered from thrombophlebitis and, theoretically at least, the clearance results could have been influenced by the absorption of streptokinase upon thrombi. However *in vitro* measurement of the uptake rates of labeled streptokinase upon human plasma clots reveal that in practice this factor probably exerted no influence upon our experimental findings. For instance labeled streptokinase was absorbed onto large plasma clots (approximate volume 400 cu. mm.) at the rate of 3.1 ± 1.1 streptokinase units per clot per 30 minutes. These determinations were made using plasma containing 15 free streptokinase units per ml. (higher concentrations than were ever attained *in vivo*) and indicate a negligible absorption compared to the large doses infused.

The results of the long term infusions with both labeled and unlabeled streptokinase appear to be of considerable significance. Since both labeled and unlabeled materials were treated similarly the influence of labeling artifact can be eliminated. Though precise estimates of plasma clearance rate could not be made, the results provide conclusive evidence that, at least within a single order of magnitude, the plasma clearance rates remained relatively stable throughout the duration of infusions lasting as long as 30 hours. This conclusion has important implications with regard to the clearance mechanisms since the 50 per cent clearance rate of a nonantigenic protein of comparable molecular weight, human albumin, has been variously determined to be 10.5 to 26.5 days (20, 21).

On both a temporal and a quantitative basis it seems necessary to postulate increased activity of the reticulo-endothelial system to explain this great difference in plasma clearance rates between the antigenic and the nonantigenic protein. Unfortunately, though all our patients showed comparable clearance rates, streptococcal infection is

so common an event during life that it is not possible to decide whether the increased ability to clear streptokinase represents an innate or an immunologically acquired quality. However suggestive evidence that this clearance mechanism may be immunologically determined comes from the observation that the streptokinase infusion rate required for the maintenance of a steady fibrinolytic state can be predicted from the initial antibody level.

These experiments suffer from the defect that the clearance of only one highly purified antigen has been studied. Other antigens might be treated in a different manner, though the general identity of these reactions with those reported to occur in animals would suggest that a phenomenon of general rather than of specialized interest has been studied.

SUMMARY

1. The plasma clearance rates of streptokinase, a protein antigenic to man, have been studied in both normal and immune man. Preparations of streptokinase, biophysically homogeneous, were trace labeled with I^{131} and were used to determine plasma streptokinase levels.

2. Intravenously injected streptokinase was cleared from the circulation at two distinct rates, the "fast" (average 50 per cent plasma clearance time 18 minutes), and the "slow" (average 50 per cent clearance time 83 minutes). The fast rate was a consequence of the combination of the antigen with its antibody, and the clearance of the complex. The "slow" rate was shown to be operative in the absence of plasma antibody and may be due to activity of the reticulo-endothelial system.

3. Specific immunization of the patient was shown to result in an increase in the proportion of antigen cleared at the fast rate. Doses of up to 10 micrograms antigen N per Kg. body weight were used in immune man, and the lack of deleterious clinical response was thought to be due to the patient's acquired ability to remove, sequestrate and destroy the antigen.

ACKNOWLEDGMENTS

This work was commenced at New York University College of Medicine, N. Y., by one of us (A. P. F.), and both the encouragement of Dr. W. S. Tillett and the assistance of Drs. A. J. Johnson and Robert Soberman are gratefully acknowledged.

Dr. Herman Eisen of Washington University kindly reviewed the manuscript and gave valuable advice.

APPENDIX

Assay for streptokinase antibody

The assay for streptokinase antibody is complicated by the presence of both plasminogen and antiplasmin in serum. Since the antigen-antibody complex is of the nonprecipitating variety, the assay must depend upon the inhibitory effect of serum, or the immunologically controlled components of serum, upon streptokinase. The use of untreated serum for this assay is inexpedient as before antigen-antibody union has occurred; plasminogen may be activated by streptokinase and later the presence of antiplasmin will interfere with the use of a biochemical indicator system.

The customary technique used for this assay, that of Christensen (11), was found to yield erroneously high values, since the period of heating at 56°C., recommended for the purpose of denaturing serum plasminogen and serum plasmin inhibitor, was unsatisfactory for this purpose. Though plasminogen became denatured in a biochemical sense, the denatured material bound streptokinase and thus the Christensen assay measured both this property and the true antibody content. Altered experimental conditions failed to obviate this anomaly and a satisfactory correction could not be applied as serum plasminogen concentration is not a fixed quantity. Accordingly streptokinase antibody assays were performed upon the isolated gamma globulin fractions of serum prepared by the technique of Lever and co-workers (12) modified only insofar as the precipitates were separated by centrifugation rather than by sintered glass filtration. This technique permitted a virtually quantitative extraction of electrophoretically homogeneous gamma globulin from the serum, which was also plasminogen free.

The success of each fractionation was judged by performing paper electrophoretic separations to demonstrate homogeneity and testing by optical density readings at 280 mμ that 9 to 13 per cent of the original serum protein was present in the isolated fraction. Some 10 per cent of samples had to be refractionated so as to comply with these criteria.

The antibody content of the serum gamma globulin fraction was estimated by a modified Christensen method (11). *1)* To each of seven tubes containing 0.4 ml. gamma globulin solution was added, by means of a microburette[4] (containing streptokinase 500 units per ml.), 1 to 50 streptokinase units; *2)* the tubes were incubated at 37°C. for 30 minutes; *3)* 0.2 ml. of a 0.25 per cent solution of Fraction III,[5] 0.2 ml. of 0.5 per cent fibrino-

[4] Micrometric Instruments, Cleveland, Ohio.

[5] Cohn Fraction III prepared from human plasma. Obtained through the courtesy of the American National Red Cross from E. R. Squibb and Sons.

gen [6] and 0.1 ml. of thrombin [7] (2 units) were added to each tube; *4*) the tubes were incubated at 37°C. and observed for lysis over a two hour period; *5*) the reciprocals of the lysis times for periods exceeding 20 minutes were plotted against the log streptokinase concentration; *6*) the resulting straight line plot was extrapolated to infinite time and the quantity of streptokinase inhibited for an infinite time by the specimen was read off from the graphic intercept; *7*) the original antibody content of the specimen was calculated and the result expressed as streptokinase units inhibited per ml. of serum.

REFERENCES

1. Coons, A. H. Labelled antigens and antibodies. Ann. Rev. Microbiol. 1954, **8**, 333.
2. Talmage, D. W., Dixon, F. J., Bukantz, S. C., and Dammin, G. J. Antigen elimination from the blood as an early manifestation of the immune response. J. Immunol. 1951, **67**, 243.
3. Francis, G. E., Hawkins, J. D., and Wormall, A. The use of radioactive isotopes in immunological investigations. 10. The fate of some intravenously injected native proteins in normal and immune rabbits. Biochem. J. 1957, **65**, 560.
4. Tillett, W. S., Johnson, A. J., and McCarty, W. R. The intravenous infusion of the streptococcal fibrinolytic principle (streptokinase) into patients. J. clin. Invest. 1955, **34**, 169.
5. Sherry, S., Fletcher, A. P., Alkjaersig, N., and Smyrniotis, F. E. An approach to intravascular fibrinolysis in man. Trans. Ass. Amer. Phycns 1957, **70**, 288.
6. Fletcher, A. P., and Johnson, A. J. Methods employed for purification of streptokinase. Proc. Soc. exp. Biol. (N. Y.) 1957, **94**, 233.
7. Johnson, A. J., Fletcher, A. P., McCarty, W. R., and Tillett, W. S. Effects in patients of intravenous infusions of purified streptokinase preparations. Proc. Soc. exp. Biol. (N. Y.) 1957, **94**, 254.
8. Wodehouse, R. P. Personal communication.
9. Eisen, H. N., and Keston, A. S. The immunologic reactivity of bovine serum albumin labelled with trace-amounts of radioactive iodine (I^{131}). J. Immunol. 1949, **63**, 71.
10. Francis, G. E., and Hawkins, J. D. The use of radioactive isotopes in immunological investigations. 11. The fate of some chemically modified protein antigens in normal and immune rabbits. Biochem. J. 1957, **65**, 570.
11. Christensen, L. R. Methods for measuring the activity of components of the streptococcal fibrinolytic system and streptococcal desoxyribonuclease. J. clin. Invest. 1949, **28**, 163.
12. Lever, W. F., Gurd, F. R. N., Uroma, E., Brown, R. K., Barnes, B. A., Schmid, K., and Schultz, E. L. Chemical, clinical, and immunological studies on the products of human plasma fractionation. XL. Quantitative separation and determination of the protein components in small amounts of normal human plasma. J. clin. Invest. 1951, **30**, 99.
13. Johnson, A. J., Fletcher, A. P., McCarty, W. R., and Tillett, W. S. The intravascular use of streptokinase. Ann. N. Y. Acad. Sci. 1957, **68**, 201.
14. Berson, S. A., Yalow, R. S., Schreiber, S. S., and Post, J. Tracer experiments with I^{131} labelled human serum albumin: Distribution and degradation studies. J. clin. Invest. 1953, **32**, 746
15. McFarlane, A. S. Labelling of plasma proteins with radioactive iodine. Biochem. J. 1956, **62**, 135.
16. Freidberg, W., Walter, H., and Haurowitz, F. The fate in rats of internally and externally labelled heterologous proteins. J. Immunol. 1955, **75**, 315.
17. Knox, W. C., and Endicott, F. C. I^{131} as an antigen label in the circulating serum of non-immune rabbits. J. Immunol. 1950, **65**, 523.
18. Melcher, L. R., and Masouredis, S. P. The in vivo stability of the I^{131} protein label of rabbit antibody in guinea pigs as determined by the quantitative precipitin reaction. J. Immunol. 1951, **67**, 393.
19. Cohen, S., Holloway, R. C., Matthews, C., and McFarlane, A. S. Distribution and elimination of ^{131}I and ^{14}C-labelled plasma proteins in the rabbit. Biochem. J. 1956, **62**, 143.
20. Sterling, K. The turnover rate of serum albumin in man as measured by I^{131}-tagged albumin. J. clin. Invest. 1951, **30**, 1228.
21. Margen, S., and Tarver, H. Comparative studies on the turnover of serum albumin in normal human subjects. J. clin. Invest. 1956, **35**, 1161.

[6] Bovine fibrinogen, 92 per cent of the nitrogen being clottable by thrombin. Kindly supplied by Dr. Kent Miller of the New York State Laboratories, New York.

[7] Parke Davis bovine thrombin.